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Lethal Granuloma Disintegration in Mycobacteria-Infected TNFRp55\(^{-/-}\) Mice Is Dependent on T Cells and IL-12\(^1\)

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Genetically susceptible, TNFRp55 gene-deficient (TNFRp55\(^{-/-}\)) mice succumb to infection with *Mycobacterium avium*. Before their death, *M. avium*-infected TNFRp55\(^{-/-}\) mice develop granulomatous lesions that, in contrast to granulomas in wild-type syngeneic mice, undergo acute disintegration. To determine the factors involved in these events, we depleted T cell subsets or neutralized the inflammatory cytokines IFN-\(\gamma\), IL-12, or TNF in TNFRp55\(^{-/-}\) mice infected i.v. with *M. avium*. Infected TNFRp55\(^{-/-}\) mice treated with a control mAb became moribund between days 26 and 34 postinfection, showing widespread inflammatory cell apoptosis within disintegrating granulomas. In contrast, TNFRp55\(^{-/-}\) mice depleted of either CD4\(^+\) or CD8\(^+\) cells after granuloma initiation stayed healthy until at least day 38 postinfection and showed no signs of granuloma destruction. Neutralization of IL-12, but not of IFN-\(\gamma\) or TNF, also protected *M. avium*-infected TNFRp55\(^{-/-}\) mice from granuloma decomposition and from premature death. Treatment with dexamethasone or with a specific inhibitor of inducible NO synthase did not prevent granuloma dissolution or death of TNFRp55\(^{-/-}\) mice. In conclusion, granuloma disintegration in TNFRp55\(^{-/-}\) mice is a lethal event that is dependent on IL-12 and that is mediated by an excess of T cells. *The Journal of Immunology*, 2000, 165: 483–492.

Granulomas are formed as a consequence of chronically persisting Ag and are thus a hallmark of all mycobacterial infections. Granulomas provide for a highly ordered juxtaposition of macrophages and T cells in such a way that antimycobacterial mechanisms may be effectively coordinated and regulated (1). On the other hand, granulomas displace and destroy parenchymal tissue, thereby determining the extent of organ pathology and, ultimately, the outcome of mycobacterial disease (2, 3).

The integrity of a developing granuloma is crucial in terms of disease progression and survival. For example, treatment of mice with an anti-TNF antiserum during the chronic stage of infection with *Mycobacterium bovis* bacillus Calmette-Guérin resulted in granuloma dissolution and extensive bacterial proliferation (4), and mice deficient in some aspect of organized mononuclear cell recruitment, such as SCID, IFN-\(\gamma\)-knockout, or TNF-knockout mice, readily succumbed to infection with *Mycobacterium tuberculosis* (5–7). In particular, the disintegration of the granuloma structure in the form of caseous necrosis and rupturing of the cavity into a bronchus is characteristic of advanced human tuberculosis (2, 3). The cellular and molecular mechanisms involved in this process have largely defied definition because of the lack of a mouse model of infection that adequately reflects the full spectrum of the immunopathology evident in humans.

Experimental infection in mice with *Mycobacterium avium*, the causative agent of the most prevalent opportunistic infection in AIDS patients, is particularly well-suited to the study of granuloma induction, maintenance, and necrosis. After i.v. infection with *M. avium*, mice develop persistent granulomatous lesions in all infected organs (8–11). Aerogenic infection with highly virulent *M. avium* strains induces a pulmonary pathology remarkably similar to that found in human tuberculosis (12).

Exploiting the *M. avium* model for the study of factors involved in the formation and maintenance of granulomas, we previously showed that granuloma development was significantly delayed in mice deficient for the TNFRp55 (13). Although these mice had similar bacterial counts in infected organs compared with immunocompetent mice, *M. avium*-infected TNFRp55\(^{-/-}\) mice all succumbed to infection. Before death, granulomatous lesions in TNFRp55\(^{-/-}\) mice acutely disintegrated, showing widespread inflammatory cell apoptosis and necrosis of both granulomatous and surrounding parenchymal tissues. Furthermore, significantly increased numbers of CD3\(^+\) cells within disintegrating lesions of TNFRp55\(^{-/-}\) mice were found, and higher levels of the proinflammatory mediators IFN-\(\gamma\), IL-12p40, and TNF were detected in organ homogenates of *M. avium*-infected TNFRp55\(^{-/-}\) mice (12, 13).

It remained unknown whether T cells and/or the mentioned key inflammatory mediators were causally involved in both the death and the granuloma disintegration occurring in TNFRp55\(^{-/-}\) mice in the course of *M. avium* infection. Therefore, we selectively depleted T cell subpopulations or neutralized proinflammatory mediators in *M. avium*-infected TNFRp55\(^{-/-}\) mice and investigated the effect of these treatments on survival, bacterial containment, and histopathology. Our results show that both granuloma disintegration and death of TNFRp55\(^{-/-}\) mice infected with *M. avium* are dependent on T cells and IL-12.

**Materials and Methods**

**Mice**

The bcg-susceptible TNFRp55\(^{-/-}\) mice used in these studies are fifth generation backcrosses of the original TNFRp55-deficient 129Sv strain (14)
onto C57BL/6 mice. TNFRp55−/− and syngeneic C57BL/6 TNFRp55+/+ mice were raised in the animal breeding facilities of Charles River Wiga (Sulzfeld, Germany). TNFRp55−/− mice on a 129Sv background (15) were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were the kind gift of Dr. U. Steinhoff (Max-Planck-Institute of Infection Biology, Berlin, Germany). All mice were serologically found to be free of Abs to the most common viral and bacterial mouse pathogens. Mice were used when they were 8–12 wk old. During the course of M. avium infection, age- and sex-matched groups of four to five mice per experimental group were housed in isolator cages under barrier conditions in the animal facilities at the Borstel Research Center.

Bacteria

M. avium, strain TMC724 (originally obtained from Dr. F. Collins, Trudeau Institute, Saranac Lake, NY), was passaged in C57BL/6 mice twice and cultured in Middlebrook 7H9 (Difco, Detroit, MI) medium supplemented with OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson, Heidelberg, Germany) to a mid-logarithmic phase. Aliquots were frozen at −70°C until they were needed. An inoculum of bacteria was prepared by thawing an aliquot and diluting it in PBS. Groups of five mice per variable experimental were infected i.v. via a lateral tail vein with indicated inocula in 0.2 ml PBS. Mice were scored as moribund and sacrificed when weight loss exceeded 25% of the body weight at the beginning of infection. Mice were anesthetized and killed at indicated time points to determine bacterial CFU in infected organs. Organs were removed aseptically, homogenized in 1 ml distilled water to determine bacterial loads by plating serial 10-fold dilutions of whole organ homogenates on nutrient Middlebrook 7H10 agar (Difco) supplemented with OADC. Bacterial colony numbers (CFU) were determined after 14–21 days incubation at 37°C in humidified air. Data are shown as mean log10 CFU counts ± SD.

The natural course of infection and the kinetics of granuloma formation and disintegration was assessed in four different experimental groups. TNFp55−/− mice infected with these strains were previously described (11, 13). All animal experiments were approved by the local ethics committee instituted by the Ministry of Nature, Environment and Forestation (Kiel, Germany).

Reagents

The following mAbs (specificities in parentheses) were used for in vivo studies: GK1.5 (anti-CD4), 2.43 (anti-CD8), 23/7 (irrelevant epitope), XMG1.2 (anti-IFN-γ, kindly provided by Dr. A. O’Garra from the DNAx Research Institute, San Diego, CA), and C 15.1 and C 15.6 (both anti-IL-6, kindly provided by the Wistar Institute, Philadelphia, PA). Abs were purified by ammonium sulfate precipitation and subsequent dialysis. The anti-CD4 mAb TN3-19.12 was a kind gift from Dr. R. Schreiber (Washington University School of Medicine, St. Louis, MO). The hamster anti-IFN-γ mAb (H22) was purchased from Genzyme (Rüsselsheim, Germany). Hamster control IgG (C 15.6, kindly provided by the Wistar Institute, Philadelphia, PA) were purified by ammonium sulfate precipitation and subsequent dialysis. Abs to the most common viral and bacterial mouse pathogens. Mice were used when they were 8–12 wk old. During the course of M. avium infection, age- and sex-matched groups of four to five mice per experimental group were housed in isolator cages under barrier conditions in the animal facilities at the Borstel Research Center.

Histology

One cranial and one caudal liver lobe per mouse were fixed in 4% formaldehyde-PBS, set in paraffin blocks, sectioned (2–3 μm), and stained using hematoxylin and eosin. In the figure legends, the original magnification of the photographic image is indicated.

Immunohistology

For the detection of CD4+ cells or CD8+ cells, frozen tissue sections were prepared using a cryostat (Frigocut E 2800; Leica, Bensheim, Germany). Four-micrometer sections were air-dried and fixed in acetone before storage at −70°C. After acetone-chloroform treatment, sections were blocked with 0.5% superoxide and incubated with mAb KT174 (anti-CD4) or KT15 (anti-CD8α). Appropriately diluted rabbit anti-rat IgG was used as a secondary Ab, and goat anti-rabbit IgG peroxidase was used as a tertiary Ab. For the detection of proliferating cells, a rabbit anti-mouse Ki-67 antiserum was used (16) with goat anti-rabbit IgG and donkey anti-goat IgG peroxidase as secondary Abs. Development was performed with 3–3′-diaminobenzidine (Sigma, Deisenhofen, Germany) and urea superoxide (Sigma), and hemalum was used to counterstain the slides. The number of granuloma-associated cells positive for a specific marker was determined by counting them in three randomly chosen 40× microscopic fields in five nonsequential liver sections per mouse (four mice per group). Data represented the means of 60 determinations ± SD.

Electron microscopy

Perfusion fixation of the liver was performed at room temperature via the vena portae with 2% glutaraldehyde/0.6% paraformaldehyde in a 0.06 M sodium cacodylate buffer (pH 7.35) for 10 min. Livers were immersed in the fixative for 72 h at 4°C, rinsed in 0.2 M sodium cacodylate buffer (pH 7.35), and postfixed with 1% osmium tetroxide in the same buffer for 2 h at room temperature. After rinsing in 2.4% sodium chloride solution, the samples were washed in 0.2 M sodium acetate buffer (pH 5.0) and block stained with 1% uranyl acetate in 0.2 M sodium acetate buffer in the dark for 30 min. After dehydration in alcohol and embedding in araldite, semithin sections (0.5 μm) were prepared and stained with azure II-methylene blue. Ultrathin sections (60 nm) were stained with lead citrate and examined in a Philips 400 electron microscope.

Cytokine ELISAs and determination of liver enzyme levels

Plasma was obtained after centrifugation of heparinized blood drawn from the posterior vena cava of anesthetized mice and stored at −70°C until further use. ELISA measurements of TNF, IL-12p40, and IFN-γ levels in the plasma were conducted as stipulated by the manufacturer (R&D Systems, Wiesbaden, Germany). Levels of alanine serum aminotransferase (ASAT) and lactate dehydrogenase (LDH) were measured in the plasma of mice using standard procedures and an automated sample analyzer in the laboratory of clinical biochemistry of the Borstel Clinical Center.

Statistics

Quantifiable data are expressed as the means of individual determinations ± SD. Statistical analysis was performed using Student’s t test, or the Welch test in case of unequal variances.

Results

Effect of T cell subset depletion on the survival of M. avium-infected TNFRp55−/− mice

The course of bacterial replication and delayed granuloma formation in M. avium-infected TNFRp55−/− mice was previously described in detail (13). TNFRp55−/− mice infected with 104 CFU M. avium TMC724 initiated granuloma formation between days 10 and 15 postinfection, whereas similarly infected TNFRp55−/− mice developed granulomatous lesions with a delay of ~5–8 days. Mononuclear cell infiltrations were more diffuse in TNFRp55−/− mice.
mice, had a higher cellularity in relation to their smaller size, and lacked the appearance of mature granulomas because they contained fewer epithelioid macrophages. With an inoculum of 10^6 CFU *M. avium*, granuloma disintegration in TNFRp55^-/- mice reproducibly occurred during the fifth week postinfection, and infected TNFRp55^-/- mice succumbed to infection before day 35.

When examined by immunohistochemistry at day 21 postinfection, T cell numbers in incipient lesions of TNFRp55^-/- mice were similar to those found in the granulomas of TNFRp55^+/- mice (Ref. 13 and data not shown). However, when the amount of CD4^+ and CD8^+ T cells in disintegrating granulomatous lesions of TNFRp55^-/- mice were compared with those present in epithelioid granulomas in TNFRp55^+/- mice at day 33 postinfection (Fig. 1), a significant increase in both subsets was evident (59 ± 6 vs 20 ± 7 granuloma-associated CD4^+ cells and 77 ± 11 vs 15 ± 4 granuloma-associated CD8^+ cells per 400× microscopic field; *p < 0.01*). The staining pattern for the proliferation-associated Ag Ki-67 showed a similar distribution as the staining pattern for these lymphocyte markers, and the number of proliferating Ki-67^+ cells was also significantly increased in lesions of TNFRp55^-/- mice at day 33 postinfection (Fig. 1).

To evaluate whether T cells were involved and, subsequently, which T cell subset was involved in granuloma disintegration, groups of five TNFRp55^-/- mice were infected with 10^6 CFU *M. avium* and treated with 0.5 mg of anti-CD4, anti-CD8, or control mAb on days 23 and 30 of infection, i.e., subsequent to granuloma establishment. Depletion efficacy was assessed by immunohistology of the liver with Abs recognizing a different epitope. Anti-CD4 treatment resulted in depletion of more than 95% of CD4^+ cells, and anti-CD8 treatment depleted ~90–95% of CD8^+ cells (data not shown).

All TNFRp55^-/- mice that had received control mAbs became moribund between days 26 and 30 of infection (Fig. 2). In contrast, TNFRp55^-/- mice that had received either anti-CD4 or anti-CD8 mAbs appeared completely healthy until day 38, when all treated TNFRp55^-/- mice were sacrificed to compare the lesions of treated surviving mice with the lesions of control mAb-treated moribund mice. TNFRp55^-/- mice receiving three injections (on days 24, 31, and 38 postinfection) of both anti-CD4 and anti-CD8 mAbs remained completely healthy until day 45, when the experiment was terminated (Fig. 2). Thus, depletion of T cell subsets significantly prolonged survival of TNFRp55^-/- mice infected with *M. avium*.

In the next step, bacterial loads in the livers, spleens, and lungs were compared between moribund control mAb-treated TNFRp55^-/- mice and T cell subset-depleted TNFRp55^-/- mice (Fig. 3). There was no significant difference between groups in any of the organs tested. Thus, depletion of T cell subsets, which prolonged survival in TNFRp55^-/- mice, did not affect bacterial loads.
loads in the time frame studied, indicating that death of TNFRp55−/− mice did not correlate with bacterial burden.

**Effect of T cell subset depletion on granuloma structure in M. avium-infected TNFRp55−/− mice**

Liver histology was examined in moribund TNFRp55−/− mice treated with control mAbs and was compared with anti-CD4- or anti-CD8-treated TNFRp55−/− mice sacrificed at day 38 postinfection. Conventional and electron microscopy was performed (Fig. 4). Control mAb-treated TNFRp55−/− mice showed massive granuloma disintegration with the characteristic hallmarks of wide-spread apoptotic cell death. Among these apoptotic cells, many macrophages could be identified by the presence of engulfed mycobacteria. In contrast, T cell subset-depleted TNFRp55−/− mice had almost normal granuloma development, showing incipient epithelioid cell differentiation and lacking signs of inflammatory cell apoptosis. In addition, granuloma integrity was fully maintained until day 45 in those TNFRp55−/− mice that had been treated with three injections (on days 24, 31, and 38 postinfection) of the combination of anti-CD4 and anti-CD8 mAbs.

Adjacent to disintegrating granulomas in control mAb-treated TNFRp55−/− mice, liver cell necrosis was evident. In accordance with this finding, TNFRp55−/− mice treated with control mAb had dramatically increased ASAT and LDH serum levels. In comparison, liver enzyme levels in T cell subset-depleted TNFRp55−/− mice did not significantly differ from those seen in C57BL/6 TNFRp55+/− mice (Table 1). Thus, prolonged survival of TNFRp55−/− mice correlated with granuloma and tissue integrity, and T cells were the major mediators of host-damaging processes in infected TNFRp55−/− mice.

To further substantiate this finding, two additional experiments were performed. First, we infected TNFRp55−/− 129Sv mice with 10⁶ CFU M. avium. These mice carry the resistant allele of the natural resistance associated macrophage protein (Nramp1) gene and are known to develop few T cell responses to intracellular infections (22, 23). These mice indeed showed a remarkable innate capacity to inhibit proliferation of the highly virulent strain TMC724 (Fig. 3) and developed only small granulomatous lesions when examined at days 38 and 56 of M. avium infection. No granuloma destruction was evident in these mice during the entire observation period (up to 84 days), and ASAT and LDH levels were comparable to immunocompetent control-infected mice sacrificed at the same time (Table 1).

Second, SCID mice deficient in T and B cells were infected with M. avium TMC724 and were treated with a neutralizing anti-TNF mAb starting on day 35 (i.e., subsequent to the delayed granuloma initiation in these mice; Ref. 18) for up to 5 wk. These mice did not show any signs of granuloma dissolution, further substantiating our interpretation that T cells are critically involved in granuloma disintegration.

**Effect of cytokine neutralization on the survival of M. avium-infected TNFRp55−/− mice**

To analyze the effect of T cell subset depletion on the levels of proinflammatory cytokines, the amounts of IL-12p40, TNF, and IFN-γ were determined in the sera of infected mice (Fig. 5). IL-12p40 levels were somewhat increased in infected TNFRp55−/− mice when compared with infected TNFRp55+/+ mice. However, T cell subset depletion had no profound effect on IL-12p40 detectable in the sera of infected TNFRp55−/− mice. IFN-γ and TNF amounts were markedly elevated in infected TNFRp55−/− mice when compared with infected TNFRp55+/+ mice. Treatment of infected TNFRp55−/− mice with anti-CD4 or anti-CD8 mAbs reduced the levels of IFN-γ and TNF to the levels found in M. avium-infected TNFRp55+/− mice (Fig. 5).

To establish whether any of these mediators were causally involved in the granuloma disintegration observed in M. avium-infected TNFRp55−/− mice, each cytokine was neutralized by i.p. injection of neutralizing doses of specific mAbs on days 25 and 32 postinfection, i.e., subsequent to granuloma establishment. Starting on day 25 postinfection, an additional group of infected TNFRp55−/− mice was s.c. treated every other day with 0.4 mg dexamethasone as a nonspecific inhibitor of inflammation.

Neutralization of TNF or IFN-γ, as well as treatment with dexamethasone, failed to increase survival times of infected TNFRp55−/− mice. All TNFRp55−/− mice thus treated died before day 37, which is similar to the results obtained with control mAb-treated TNFRp55−/− mice (Fig. 6). In contrast, mice treated for 2 wk with a combination of two mAbs specific for IL-12p40 survived until day 38 when they were sacrificed to compare their liver histopathology with that of moribund control mAb-treated
FIGURE 4. Histopathology and electron microscopy of lesions in the livers of *M. avium*-infected mice. For experimental procedures, see Fig. 3. a and c, Well-organized granulomas in TNFRp55+/− mice composed of epithelioid cells with large, round nuclei and granular endoplasmatic reticulum (open arrow) and mycobacteria in phagocytic vacuoles (filled arrow). b and d, Poorly organized, disintegrating granulomas in TNFRp55−/− mice showing numerous apoptotic cells with fragmented nuclei containing condensed chromatin and apoptotic bodies (filled arrows). e and g, Well-defined granulomas in TNFRp55−/− mice depleted of CD4+ T cells. Granulomas contain epithelioid cells with round nuclei and granular endoplasmatic reticulum (open arrow). Filled arrow indicates mycobacteria within phagocytic vacuoles. f and h, Well-structured granulomas in TNFRp55−/− mice depleted of CD8+ T cells. Granulomas contain epithelioid cells with round nuclei and granular endoplasmatic reticulum (open arrow). a, b, e, and f, Hematoxylin and eosin stain (magnification ×64). c, d, g, and h, Electron micrographs (magnification ×2800).
TNFRp55−/− mice. Infected TNFRp55−/− mice receiving three injections of the anti-IL-12p40 mAbs (on days 24, 31, and 38 postinfection) remained completely healthy until the experiment was terminated on day 45 (Fig. 6). Thus, survival of M. avium-infected TNFRp55−/− mice was markedly prolonged by effective neutralization of IL-12.

Again, CFU counts in the treatment groups were compared. Dexamethasone treatment resulted in a significant increase in bacterial CFU counts in the liver, but all other groups showed practically identical, very high bacterial loads in the liver and spleen (Fig. 7). Therefore, survival did not correlate with different bacterial organ loads in the different mouse groups.

**Effect of cytokine neutralization on liver histology of M. avium-infected TNFRp55−/− mice**

Histopathological examination revealed that anti-IL-12p40-treated TNFRp55−/− mice had compact granulomas showing epithelioid cell differentiation, whereas immature granulomatous lesions in TNFRp55−/− mice treated with anti-IFN-γ, anti-TNF, or control mAb had completely disintegrated and were again characterized by an abundance of apoptotic cells (Fig. 8). Granulomas in infected, dexamethasone-treated moribund TNFRp55−/− mice were completely necrotic, whereas the surrounding liver tissue appeared less affected.

Immunohistological analysis showed that fewer CD3+ cells accumulated in differentiating granulomatous lesions of anti-IL12p40-treated TNFRp55−/− mice than in disintegrating lesions of control mAb-treated TNFRp55−/− mice. Decreased recruitment after anti-IL-12p40 treatment was particularly pronounced for CD8+ cells and at day 45 postinfection.

The extent of granuloma disintegration and subsequent tissue necrosis in the different groups was quantitated using ASAT and LDH levels in the sera of mice. Only anti-IL-12p40-treated mice had low ASAT and LDH levels, results similar to those of infected TNFRp55−/− controls, whereas infected control mAb-treated TNFRp55−/− mice or infected mice which had received anti-IFN-γ had high levels of ASAT and LDH (Table II). TNFRp55−/− mice treated with anti-TNF or dexamethasone also showed reduced ASAT and LDH levels compared with those of untreated TNFRp55−/− mice; however, ASAT and LDH levels were still markedly elevated compared with those of TNFRp55−/− mice treated with the anti-IL-12p40 mAbs (Table II).

In conclusion, prolonged survival of M. avium-infected anti-IL-12p40-treated TNFRp55−/− mice correlated with maintained granuloma integrity, whereas granuloma disintegration proceeded unaltered in infected TNFRp55−/− mice treated with anti-TNF or anti-IFN-γ and was always followed by premature death.

Because the inducible form of NO synthase (iNOS) was previously shown to be up-regulated in M. avium-induced granulomas (13, 21), the possibility was considered that NO might be responsible for the tissue damage in infected TNFRp55−/− mice. Therefore, TNFRp55−/− mice were infected and treated with the selective inhibitor of iNOS, l-NIL. However, l-NIL-treated TNFRp55−/− mice did not survive longer than untreated infected TNFRp55−/− mice, and granulomas of l-NIL-treated mice disintegrated with the characteristic histomorphology also evident in infected TNFRp55−/− mice left untreated. In accordance, ASAT and LDH serum levels were similarly high in both groups of mice.

**Discussion**

The outcome of mycobacterial diseases is intrinsically linked to granuloma formation and maintenance of granuloma integrity (2,
FIGURE 6. Survival of M. avium-infected TNFRp55^+/− mice treated with cytokine-neutralizing mAbs. TNFRp55^+/− mice were infected with 10^6 CFU M. avium and treated with i.p. injections of the indicated neutralizing mAbs on days 25 and 32 postinfection or with s.c. injections of dexamethasone every other day after day 25 postinfection. Data represent the percentage of surviving mice per group in the course of M. avium infection (four to five mice per group; eight mice in the control mAb-treated group). Data are from one experiment of two, which gave qualitatively identical results.

3, 24). Using i.v. infection with M. avium in the mouse as a model system to study the factors governing granuloma integrity, we have previously established that a lack of TNFRp55 signaling results in a disregulated granulomatous response, namely granuloma disintegration and tissue necrosis (12, 13). We now demonstrate that T cells and IL-12 are crucially involved in inflammatory cell apoptosis and granuloma decomposition in M. avium-infected TNFRp55^+/− mice. In fact, prevention of granuloma disintegration by neutralizing IL-12 or depleting T cell subsets significantly prolonged survival of M. avium-infected TNFRp55^+/− mice. Our results suggest a hitherto underestimated detrimental and pathology-promoting role of T cells in mycobacteria-induced lesions.

The pivotal role of both CD4+ and CD8+ T cells in antmycobacterial protection is well-established and is thought to involve the enhanced production of IFN-γ (6, 9). Regarding M. avium infection, no role was found for CD8+ cells in this respect (9, 25). Granuloma formation in response to M. avium infection proceeds in an accelerated fashion in the presence of CD4+ cells (11, 18); again, CD8+ T cells were demonstrated not to be involved in this process in immunocompetent mice (11, 25).

However, in a disregulated inflammatory response, such as that evident in M. avium-infected TNFRp55^+/− mice, both subsets apparently contribute to granuloma disintegration. The cause for the increased presence of both CD4+ and CD8+ T cells in disintegrating granulomatous lesions of TNFRp55^+/− mice is not entirely clear. One explanation might be that during the course of infection in TNFRp55^+/− mice, TNFRp55-mediated apoptosis occurs in T cells recruited to or actively dividing in the granuloma. This mechanism would normally regulate the number of T cells present within the lesion, as described in murine autoimmune encephalomyelitis (26). A lack of TNFRp55-mediated T cell apoptosis would result in the enhanced accumulation of T cells, particularly under conditions in which the proliferation of lymphocytes in situ is greatly enhanced, as demonstrated in this study for the lesions in infected TNFRp55^+/− mice.

The lethal outcome of M. avium infection in TNFRp55^+/− mice investigated in this study is in apparent contrast to another report (10). However, the investigators in that study made use of TNFRp55^+/− mice on a 129Sv/BL6 background, which may be assumed to carry the resistant allele of Nramp1. Our own experiments confirmed that genetically resistant TNFRp55^+/− mice infected with M. avium do not show granuloma disintegration. Because resistant mice develop only minimal T cell responses to intracellular infections (22, 23), we take this as corroborating evidence that T cells are indeed necessary for the lethal event of granuloma dissolution. Although the inflammatory response in SCID mice is not equivalent to that in immunocompetent mice (18), our experiments in which chronic neutralization of TNF in M. avium-infected SCID mice also did not affect granuloma development tend to further substantiate our interpretation that lethal granuloma disintegration is T cell-dependent.

Most M. avium isolates readily induce IL-12p40 in vitro (27), and we found high levels in the sera of both TNFRp55^+/− and TNFRp55^+/− mice. Neutralization of IL-12 led to prolonged survival of M. avium-infected TNFRp55^+/− mice, and granulomatous lesions in these mice contained fewer CD3+ cells, specifically fewer CD8+ cells, and did not disintegrate. IL-12 was previously shown to promote lymphocyte recruitment into developing granulomatous lesions (28), and this effect may have critically contributed to lesion development in TNFRp55^+/− mice. On the other hand...
hand, IL-12p70 is also known to activate Th1 cells (29), and IL-12R interaction in vitro with IL-12p40 homodimer resulted in enhanced Th1 development and increased IFN-γ secretion from CD8+ T cells (30). Moreover, IL-12p40 levels positively correlated with disease activity in multiple sclerosis (31, 32). In line with these reports, we hypothesize that IL-12 is involved in both the recruitment of T cells and their activation within the developing lesion.

The decomposition of granulomas in infected TNFRp55−/− mice always started with conspicuous apoptosis of inflammatory cells within the lesions. Macrophages seemed to be particularly affected, as judged by the frequent presence of mycobacteria within apoptotic cells, although we did not specifically investigate whether other cells, such as T cells or adjacent hepatocytes, also underwent apoptosis. However, apoptosis was strikingly absent when T cells were depleted.

Increased numbers of T cells in granulomatous lesions of TNFRp55−/− mice were associated with dramatically higher amounts of IFN-γ in the sera of these mice, and depletion of T cells resulted in reduced IFN-γ levels in the sera of TNFRp55−/− mice. Therefore, one might argue that T cell-derived IFN-γ was the principal inducer of inflammatory cell apoptosis and subsequent tissue necrosis, as was previously shown in the model of gastrointestinal infection with Toxoplasma gondii (33, 34). Although we used, in separate experiments, two different neutralizing mAbs against IFN-γ, both of which had previously been shown to be highly effective in vivo (9, 18), we were unable to demonstrate a role for IFN-γ in granuloma disintegration in infected TNFRp55−/− mice. This finding was corroborated by the results obtained with dexamethasone treatment, which reduced serum IFN-γ in infected TNFRp55−/− mice almost to background levels, but it was ineffective at inhibiting granuloma necrosis. Interestingly, and in contrast to results obtained in the oral infection model with T. gondii in genetically susceptible mice (35), inhibition of iNOS also did not affect the immunopathology observed in TNFRp55−/− mice infected with M. avium.

T cells are known to have the potential for autoreactivity resulting in tissue destruction (36, 37). In experimental models of autoimmune diseases, Fas/Fas ligand (FasL) interactions and membrane TNF-TNFRp75 interactions were described as important mechanisms for the induction of apoptosis by CD4+ T cells (38–40). Cytotoxic T cells were shown to destroy infected target cells via either a Fas/FasL interaction or via a granule-dependent mechanism (41). The exact mechanism by which T cells contribute to inflammatory cell apoptosis and tissue necrosis in infected TNFRp55−/− mice remains to be determined. Because TNF neutralization did not affect M. avium-induced lethality in TNFRp55−/− mice, we consider the TNF/TNFRp75 pathway least likely. However, we cannot formally rule out that signaling through the TNFRp75 did occur in our experiments, because membrane-bound TNF may have been suboptimally affected by our anti-TNF treatment. Whether IL-12, in addition to promoting recruitment of lymphocytes into the developing granuloma (28),

FIGURE 8. Liver histopathology in M. avium-infected TNFRp55−/− mice treated with cytokine-neutralizing Abs. For experimental procedures, see Fig. 7. a, TNFRp55−/− mice, treated with control mAbs. Disintegrating and necrotizing granulomatous lesions. b, TNFRp55−/− mice, treated with anti-IL-12p40 mAbs. Well-structured granulomas with epithelioid macrophages. c, TNFRp55−/− mice, treated with anti-IFN-γ mAb. Disintegrating lesions with numerous apoptotic cells. Livers of TNFRp55−/− mice treated with anti-TNF mAb looked identical. d, TNFRp55−/− mice, treated with dexamethasone. Hematoxylin and eosin stain; magnification ×64. Arrows indicate apoptotic cells. X, Necrotic areas.
also directly up-regulates the expression of Fas/FasL or of the perforin/granzyme system is presently not known.

In conclusion, this study shows that lack of TNFRp55 signaling in the course of M. avium infection results in a hyperinflammatory and ultimately fatal response in which IL-12 promotes the recruitment of T cells, which induce inflammatory cell apoptosis leading to granuloma disintegration. Therefore, TNFRp55-mediated signaling appears to be an integral part of a feedback loop between macrophages and T cells that is normally in place to control and regulate the potentially detrimental T cell-mediated inflammatory response to mycobacterial infection.

The integrity of granulomas is a critical determinant of the outcome of mycobacterial diseases, and apoptosis and necrosis are common events in granulomas associated with human tuberculosis (2, 3, 24, 42, 43). The mycobacteria-infected TNFRp55−/− mouse may prove useful in defining suitable molecular targets for therapeutic interventions aimed at reducing tissue-destructive and life-threatening immunopathology caused by an excess of T cells. Certainly, our findings obtained in gene-deficient mice cannot be directly extrapolated to the immunopathology that occurs in immunocompetent individuals with mycobacterial or other intracellular infections. However, recent results from other experimental infections in TNFRp55−/− mice suggest that the observed absence of adequate lesion control is not unique to infections with a particular pathogen because failure to signal through the TNFRp55 also resulted in failure of lesions to regress once Leishmania major or Rhodococcus equi was eliminated (44). The use of TNFRp55−/− mice has thus not only uncovered a unique role of TNF in the adequate maintenance and resolution of inflammatory lesions, but it also highlights the potentially detrimental and pathology-promoting function of T cells that develops in the course of different types of infections and must be effectively harnessed.

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References


